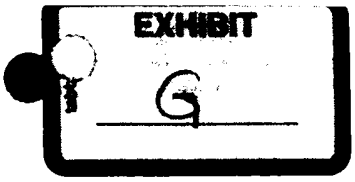

Cultivation of the ubiquitous marine bacterium SAR11

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The alpha proteobacterium SAR11 was among the first new organisms discovered when cultivation independent approaches based on ribosomal RNA gene cloning and sequencing were applied to survey microbial diversity in natural ecosystems[1]. It accounts for 26% of all ribosomal RNA genes detected in seawater, and has been found in every pelagic marine bacterioplankton community studied by these methods[2]. SAR11 is representative of a pervasive problem in microbiology: despite its ubiquity, it has defied cultivation efforts. Genetic evidence suggests that diverse uncultivated microbial taxa dominate most natural ecosystems[3-5], prompting widespread efforts aimed at elucidating the geochemical activities of these organisms without the benefit of cultures for study[6,7]. Here we report the first isolation of SAR11. Eighteen cultures were obtained by means of newly developed high throughput procedures for isolating cell cultures by the dilution of natural microbial communities into very low nutrient media. The volume of these cells, ca. 0.01 μm^3, places them among the smallest free-living cells in culture.

In an effort to isolate some of the ubiquitous uncultivated bacteria and archaea that dominate marine bacterioplankton communities[2], fresh Oregon coast seawater samples were inoculated into microtiter dish wells by dilution, such that on the average each well received 22 microbial cells. Media consisted of sterile Oregon coast seawater supplemented with phosphate and ammonium, or phosphate, ammonium and a defined mixture of organic carbon compounds. The technique of isolating cells by dilution into sterilized natural waters or other dilute media has been used previously[8-11]. This approach

takes advantage of the fact that substrate concentrations and cell numbers in natural waters are typically about three orders of magnitude less than in common laboratory media. The approach we used to isolate SAR11 was similar but involved modifications to a microtiter dish format and the use of a newly developed procedure for making arrays of cell cultures on microscope slides, coupled with fluorescence *in situ* hybridization (FISH)[10,11]. These procedures were designed to increase the rate at which cultures could be obtained and identified. In this experiment, 288 dilution cultures of 1 ml volume were inoculated and screened.

After incubation at 15 °C for 23 days in either the dark or under a 14:10 light:dark cycle (approximately 400 μmol m^{-2} s^{-1} irradiance), culture wells were tested for replicating cells by arraying cells on polycarbonate membranes. The limit of detection with this method is approximately 2×10^3 cells ml^{-1}, and it requires only 100 μl (ca. 200 cells). Cultures that tested positive by staining of arrays with the DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI)[12] were further screened by FISH with a set of four fluorescently-labeled oligonucleotide probes specific for SAR11 16S rRNA (Fig. 1). Ten axenic cultures and 8 mixed cultures of SAR11 were preliminarily identified. Isolates were obtained in cultures incubated in the dark and under a light:dark diel cycle, as well as in the medium containing natural organic carbon and medium supplemented with a defined mixture of carbon compounds. Isolate identity was confirmed by a combination of ribosomal RNA gene sequencing and restriction fragment length polymorphism (RFLP) analysis of PCR amplified 16S rDNA. The ten axenic isolates of SAR11 yielded identical RFLP patterns, and 16S rDNA sequence analysis of three isolates also showed them to be identical.

The nearly complete 16S rDNA sequence from one SAR11 isolate (strain HTCC1013) was almost identical (>99% similar) to rRNA-gene-containing clones

recovered from coastal seawater of Arctic Alaska[13], the coast of Plymouth, U.K.[14] (Fig. 2), and the Santa Barbara Channel[15] (data not shown). Comparative sequence analysis of 16S rRNA genes shows SAR11 and relatives to be a rapidly evolving, deeply branching cluster of the alpha subclass of Proteobacteria (Fig. 2). Members of this group show less than 82% sequence similarity to cultivated members of the alpha Proteobacteria. Since its original discovery in the Sargasso Sea, members of the SAR11 cluster have been recovered in every 16S rRNA gene clone library constructed with universal or bacterial PCR primers from marine prokaryotic plankton samples, including coastal and near-shore waters[15,16], marine samples from a depth of 3000 m[17], and freshwater lakes[18,19].

The growth constant for the isolates was 0.40-0.58 d^{-1}. While this rate of cell division is low in comparison to values typical of cultivated bacteria, it is not unlike the measured growth rates of marine bacterioplankton communities in nature, which vary from 0.05-0.3 d^{-1} (ref. 20).

The size and morphology of the isolates was identical to SAR11 cells in natural populations observed by fluorescence *in situ* hybridization. SAR11 cells are crescent shaped (vibrioid). Size estimates made by transmission electron microscopy on a culture of HTCC1014 cells indicate that it is one of the smallest cells known (Fig. 3). Cell length varied from 0.37-0.89 μm, while the average cell diameter was measured at 0.12 to 0.2 μm. The size uncertainties of these estimates arise from natural variation in cell length associated with cell division, and uncertainty about whether a lightly staining structure observed on the outwardly curved lateral surface of cells is enclosed by membrane (Fig. 3). For a cell of 0.4 x 0.2 μm, which is a conservative estimate of the size of a SAR11 cell emerging from division, the cell volume is about 0.01 μm^3. This estimate is approximate, since the cells were measured after fixation in glutaraldehyde, which may have caused shrinkage.

Reliable data on the sizes of the smallest cells are scant. One report places the diameter of the smallest cultured microorganisms (mycoplasmas) at 0.34 μm (0.021 μm^3), which is about twice the size of a SAR11 cell[21]. However, some measurements suggest that *Thermoplasma* sp. may be considerably smaller[22]. The theoretical lower size limit for an autonomous cell has been estimated from a list of essential macromolecular components identified by comparative genomics[23]. SAR11 cells are 1.4 fold larger than this minimum estimate. By way of comparison, an *E. coli* cell of 1.3 × 4 μm occupies a volume of about 4.7 μm^3, 470 times the volume of SAR11. Our measurements were made on dividing populations of cells that were fixed in aldehydes, factors which respectively may increase or decrease the apparent size of cells.

Regardless, the small size of SAR11 is noteworthy, particularly considering that this organism is wonderfully well adapted to a life of autonomous replication. The success of this cultivation approach with SAR11 may be attributed to a number of factors, including the use of pristine seawater as a medium, the relatively large number of cultures screened, and the low growth rate detection threshold of the procedure. Access to SAR11 cells in culture will provide an unusual opportunity for genome sequence analysis of an organism that has global biogeochemical significance and can be manipulated in culture. It may also provide insight into the adaptations of cells to very low nutrient systems, and, because of the extraordinarily small size, the study of SAR11 should also refine our understanding of the minimal macromolecular machinery required for autonomous cellular replication.

Methods

Sample collection and culturing. A water sample was collected in a 2 litre Niskin bottle fitted with a Teflon-coated spring from a depth of 10 m at station NH15, 15 miles west of Yaquina Head, Oregon on the Newport Hydrographic line (44° 39.1' N x 124° 24.7' W).

The bottle was immediately stored in a cooler with cold packs to maintain a temperature similar to surface water at this station (12.0 °C) until further laboratory processing (3 h from sample collection). We employed two types of media: sterile coastal Pacific Ocean seawater amended with 1.0 µm NH_4Cl and 0.1 µM KH_2PO_4, and the same medium amended with 0.001% (w/v) D-glucose, D-ribose, succinic acid, pyruvic acid, glycerol, and N-acetyl glucosamine; 0.002% (v/v) ethanol; and Va vitamin mix at a 10^{-4} dilution of stock[24].

Fluorescent Microscopy. For the identification of SAR11 containing cultures by FISH, 100-200 µl from each culture well was fixed with 0.2-µl filtered paraformaldehyde (2% final concentration) and filtered onto 0.2-µm polycarbonate membrane filters. FISH was performed essentially as described[25], with the following modifications (R. Morris, unpublished data). Hybridization reactions were performed at a temperature of 35 °C for 3 to 12 h duration in hybridization buffer consisting of 900 mM NaCl, 20 mM Tris (pH 7.4), 0.01% (w/v) sodium dodecyl sulphate (SDS), 15% formamide and four Cy3 labeled oligonucleotide probes targeting SAR11 ribosomal RNA at a final concentration of 2 ng µl^{-1} each. Optimal hybridization stringency was achieved by washing the membranes for two 15 min intervals at 55 °C in 150 mM NaCl, 20 mM Tris (pH 7.4), 6 mM ethylenediaminetetraacetic acid (EDTA) and 0.01% SDS. After mounting filters in Citifluor (Ted Pella), Cy3-positive and DAPI-positive cells were counted for each field of view[26] using a Leica DMRB epifluorescence microscope equipped with a Hamamatsu ORCA-ER CCD digital camera, filter sets appropriate for Cy3 and DAPI, and Scanalytics IPLab v3.5.5 scientific imaging software.

Electron Microscopy. Cells were initially concentrated by centrifugation on Vivascience Vivaspin 500 ultrafiltration concentrators and re-suspended in sterile seawater containing 0.5% glutaraldehyde. After centrifugation of concentrated cell suspensions onto

Formvar-coated copper grids, cells were prepared for imaging by either negative staining with 2% uranyl acetate (pH 4.0), or shadowing with gold platinum. Fluoresbrite beads (0.514 ± 0.015 m diameter) were included in the preparations as an internal size standard.

Phylogenetic analysis. Genomic DNA was isolated from 200 l of culture using a Qiagen DNeasy Tissue kit after two freeze-thaw cycles. Due to the small amount of starting material, a semi-nested PCR reaction was required to obtain sufficient product for further characterization. Ribosomal DNAs were initially amplified in a 50 l PCR reaction using the primer pair 27F-1492R for 30 cycles. One l of this product was used as template for a second 50 l reaction using 27F-1406R (DNA sequencing) or 519F-1406R (RFLP). PCR products were cleaned with a Qiagen QiaQuick PCR purification column and sequenced on an ABI 377 automated sequencer. Sequences were aligned with a database of over 11,500 SSU rDNA sequences maintained with the ARB software package[27]. Phylogenetic analyses were performed with the program PAUP* 4.0 beta 8 (ref. 28), and included 951 unambiguously aligned nucleotide positions. The tree topology was inferred by maximum likelihood employing a heuristic search and tree-bisection-reconnection (TBR) branch swapping options, a transition/transversion ratio estimated from the data (1.4259:1), and nucleotide frequencies estimated from the data. Bootstrap proportions from 1000 resamplings were determined using evolutionary distances calculated with the Kimura 2-parameter model for nucleotide change, a transition/transversion ratio estimated from the data, and neighbour-joining.

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Correspondence and requests for materials should be addressed to S.J.G. (e-mail: steve.giovannoni@orst.edu). **The sequence reported in this study has been deposited in GenBank under accession number xxxxxxxx.**

Figure Legend

Figure 1 Fluorescent *in situ* hybridization of axenic SAR11 strain HTCC1014 cells. Identical field viewed by: **a**, epifluorescence microscopy with cells stained with the DNA-specific dye DAPI; and **b**, epifluorescence after hybridization with four Cy3-labelled oligonucleotide probes targeting SAR11 cells. Scale bar is 1 μm.

Figure 2 Phylogenetic relationships between strain HTCC1014 and representatives of the SAR11 cluster and alpha Proteobacteria inferred from 16S rRNA gene sequence comparisons. The Gram-positive bacteria *Bacillus subtilis* and *Marinococcus halophilus* were used as outgroups. Bootstrap proportions over 70% that supported the branching order are shown. The scale bar corresponds to 0.05 substitutions per nucleotide position. Also included in the analysis were: Gamma Proteobacteria, *Alteromonas macleodii* and *Marinobacter*

hydrocarbonoclasticus; Beta Proteobacteria, *Methylophilus methylotrophus* and *Polynucleobacter necessarius*.

Figure 3 Electron micrographs of SAR11 isolate HTCC1014. The latex beads have a diameter of 0.514 μm. **a**, transmission electron micrograph of shadowed cells with the typical SAR11 morphology. **b**, transmission electron micrograph of a negatively stained cell.

Figure 1
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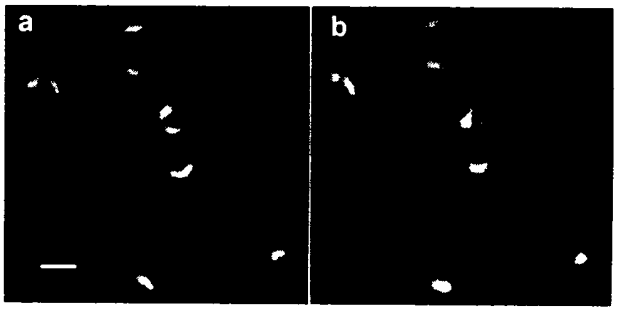

Figure 2
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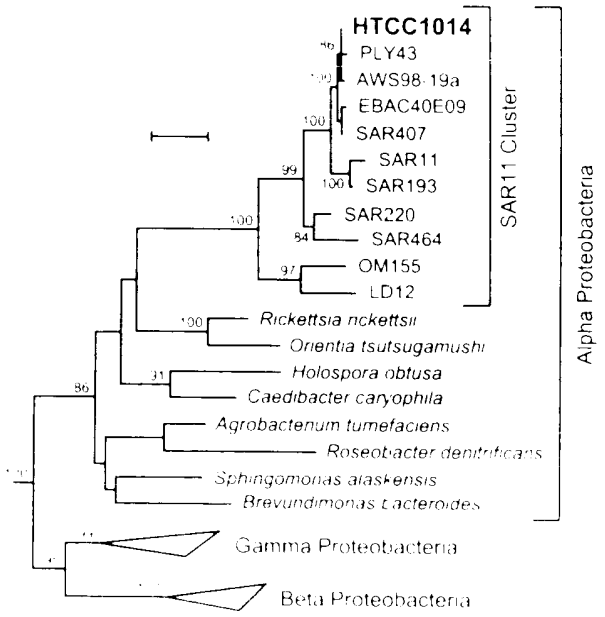

Figure 3
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